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(57) Abstract

This invention relates to the field of Bovine Viral Diarrhea Virus (BVDV), and vaccines for the treatment thereof. This invention describes the preparation of live, attenuated Bovine Herpesvirus type 1 (BHV-1) as a virus, vaccine and vector for expression of BVDV antigens. A BVDV cDNA clone containing sequences corresponding to glycoprotein gp53 is inserted into an inactivated BHV-1 virus.

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VIRAL VECTOR WITH BOVINE VIRAL DIARRHEA VIRUS (BVDV) ANTIGENS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of Bovine Viral Diarrhea Virus (BVDV), and vaccines for the treatment thereof.

Information Disclosure

van Zijl, M. *et al*. Live Attenuated Pseudorabies Virus Expressing Envelope Glycoprotein E1 of Hog Cholera Virus Protects Swine against both Pseudorabies and Hog Cholera, *Journal of Virology*, Vol. 65, No. 5, pp. 2761-2765 (1991). U.S. patent 4,703,011, Kit, M., and Kit, S., Thymidine Kinase Deletion Mutants of Bovine Herpesvirus-1, issued 27 October 1987. U.S. patent 4,824,667, Kit, M., and Kit, S. Thymidine Kinase Deletion Mutants of Bovine Herpesvirus-1, Vaccines Against Infectious Bovine Rhinotracheitis Containing Same and Methods for the Production and Use of Same, issued 25 April, 1989. Collett, M.S., *et al.*, Proteins Encoded by Bovine Viral Diarrhea Virus: The Genomic Organization of a Pestivirus, *Virology*, Vol. 165 pp. 200-208 (1988). Collett, M.S., *et al.*, Molecular Cloning and Nucleotide Sequence of the Pestivirus Bovine Viral Diarrhea Virus, *Virology*, Vol. 165 pp. 191-199 (1988).

Background

Bovine viral diarrhea virus (BVDV) is a Pestivirus belonging to the family of the Flaviviridae. It causes a number of different conditions in sheep, goats, and especially cattle. The symptoms depend upon the age, physiological and virological state of the animal. In young susceptible calves and young adults it causes a disease which is characterized by high morbidity and low mortality. The symptoms can include fever, depression, occulo-nasal discharges, diarrhea and occasionally oral ulcerations. Apart from these primary effects the virus also causes immunosuppression. Although primary BVDV infections are normally relatively mild, the virus may potentiate or enhance the pathogenicity of other co-infecting microorganisms.

In older or susceptible animals, BVDV causes similar symptoms to those described above for younger susceptible calves. In addition, in pregnant animals the virus has the ability to cross the placenta and infect the fetus. The outcome of this infection depends upon the age of the fetus and whether it is at a stage where its immune system is fully competent. The possible outcome of infections include fetal

reabsorption, abortion, mummification, congenital defects, birth defects, calves born which are persistently infected with BVDV and completely normal calves. Calves born which are persistently infected with BVDV, represent the most important segment of this BVDV pathogenesis complex. Persistently infected animals shed large amounts of virus into their environment which can infect susceptible animals. Furthermore, even though persistently infected animals are immunotolerant to the virus which infected them in utero, they do develop disease when infected with other closely related BVDV biotypes. These infections are characterized by low morbidity (because relatively speaking there will not be many pregnant animals infected at the right time during pregnancy to produce BVDV persistently infected normal calves), but high mortality. This disease syndrome is known as mucosal disease and often manifests itself as a peracute condition with calves dying of a profuse watery diarrhea which contains large amounts of fresh blood.

The importance of this virus and it's widespread presence in the cattle population has led to the development of many vaccines in the attempt to try to prevent BVDV infection. These vaccines have been built on the traditional concepts of inactivation or attenuation but, because of the behavior of BVDV, they have many significant drawbacks.

It is generally accepted that inactivated vaccine preparations are not as effective as attenuated live vaccines. Inactivated antigen from inactivated vaccine preparation undergoes exogenous processing. After injection into the animal the antigen becomes part of the animal's soluble protein mileau. The antigen enters antigen presenting cells through pinocytotic mechanisms and this usually produces antibodies. Unfortunately, because antibodies cannot gain entry into cells, they normally only interrupt viral life cycles when mature virus is released from the cell. On the other hand, antigen from live virus which replicates inside cells, undergoes endogenous processing and this mechanism produces the preferred cell mediated immune responses. Cell mediated immune responses can recognize cells infected with viruses and have the potential of interrupting the virus life cycle at a much earlier stage. Cell mediated responses are thus thought to be extremely important in the immunological defense to many viral infections.

Because of the cell mediated response, attenuated live products such as vaccines should induce good cell mediated responses. With BVDV, attenuation of the virus to produce the live vaccine does not always prevent that vaccine virus from causing the immunosuppression normally associated with field isolates. Roth J.A.

and Kaeberle M.L., Suppression of Neutrophil and Lymphocyte Function Induced by a Vaccinal Strain of Bovine Viral Diarrhea Virus With or Without the Administration of ACTH, *American Journal of Veterinary Research*, Vol. 44 pp. 2366-2372 (1983). The failure of the vaccine to stop the immunosuppression response creates a serious drawback to the vaccine. An animal owner may be vaccinating animals to protect against a disease but because of the properties of the vaccine the owner provides an opportunity for other diseases to afflict the animals. This forces the owner to use inactivated BVDV vaccines, which because of the way in which the immune system operates, are not particularly effective.

In summary, inactivated vaccines are safe but not particularly effective while the attenuated live vaccines are more effective but under certain conditions may not be very safe.

This invention combines the effectiveness of the attenuated live vaccines with the safety of the inactivated vaccines. Bovine herpesvirus type 1(BHV-1) is another major pathogen of cattle which produces respiratory disease. Thus, in common with BVDV, BHV-1 also replicates at a mucosal surface. We take the gene which codes for gp53, a major glycoprotein of the BVDV virus and against which the host produces substantial immune responses, and express it in bovine herpes virus -1 (BHV-1), this recombinant virus (BHV/BVDVgp53) is used as a vaccine against BVDV. Donis, R.O. and Dubovi, E.J., Glycoproteins of Bovine Viral Diarrhoea-Mucosal Disease Virus in Infected Bovine Cells, *Journal of General Virology*, Vol. 68, pp. 1607-1616 (1987) and Magar, R., *et al.*, Bovine Viral Diarrhea Virus Proteins: Heterogeneity of Cytopathogenic and Noncytopathogenic Strains and Evidence of 53K Glycoprotein Neutralization Epitope, *Veterinary Microbiology*, Vol. 16, pp. 303-314. Cited references are incorporated herein by reference.

SUMMARY OF THE INVENTION.

A replicating nonpathogenic virus, for preventing disease caused by Bovine Viral Diarrhea Virus (BVDV), where said replicating nonpathogenic virus comprises: a gene or gene combination taken from a BVDV virus, and said replicating nonpathogenic virus functionally expresses said gene or gene combination. Embodiments of this invention include the following: A virus where said replicating nonpathogenic virus is attenuated, is selected from attenuated Bovine Herpes Virus type 1 (BHV-1), attenuated adenoviruses, attenuated bovine mammillitis virus, attenuated bovine papillomavirus, or attenuated pseudorabies virus. A virus where said replicating nonpathogenic virus is attenuated and contains and expresses any

combination of the following genes: the genes that code for gp48, gp25, p14 capsid protein, p20 N-terminal protease and p125/p80 protein. A virus where the attenuation is created by making the thymidine kinase (tk) gene nonfunctional.

A virus where a signal peptide is inserted preceeding the gene or gene combination that codes for gp53 in said Bovine Herpes Virus type 1 (BHV-1). A virus where said gene that codes for gp53 is inserted into the inactivated thymidine kinase (tk) gene site. A virus where the functionally expressing gene or gene combination, used to create the virus, comprises a recombined plasmid with intact viral DNA, said plasmid comprising: a) a BHV-1 genomic DNA fragment containing the thymidine kinase (tk) gene and having a deletion to the thymidine kinase (tk) gene, b) a promoter/polyadenylation signal inserted in the thymidine kinase (tk) gene deletion, c) a signal peptide gene sequence preceding a gp53 gene or gene combination all of which is inserted between the promoter and the polyadenylation signal. A virus where said plasmid is made from a plasmid having the characteristics of plasmid pHAS4. A virus where said signal peptide gene sequence is taken from any well characterized signal peptide sequences such as any of the thirty-nine examples of well characterized signal peptide sequences found in Perlman, D., et al., *J. Mol . Biol.* Vol. 167 pp. 391-409 (1983), incorporated by reference. A virus where said signal peptide gene sequence is taken from Psuedorabies Virus gIII gene (PRV) and/or Bovine Growth Hormone (BGH).

A virus where a plasmid is selected from the following plasmids, a) pBHVtkex-1::BGH/p53; b) pBHVtkex-1::gIII/p53; c) pBHVtkex-3::BGH/p53; or d) pBHVtkex-3::gIII/p53. A virus that produces the product of a functionally expressing gene or gene combination is selected from one of the following viruses, T11-3, T11-6, or T11-8. A virus where the functionally expressing gene or gene combination, used to create the virus, comprises a recombined plasmid with intact viral DNA, said plasmid comprising: a) a BHV-1 genomic DNA fragment containing the thymidine kinase (tk) gene and having a deletion to the thymidine kinase (tk) gene, b) a promoter/polyadenylation signal inserted in the thymidine kinase (tk) gene deletion, c) a gp53 gene or gene combination inserted between the promoter and the polyadenylation signal. A virus where the plasmid is pBHVtkex-3::p53. A virus selected from one of the following viruses, T2-3#3 or T2-2#5. A vaccine for preventing disease caused by Bovine Viral Diarrhea Virus (BDVD) comprising a pharmaceutically effective amount of the viruses described herein and a carrier.

A vaccine as described above for preventing disease caused by Bovine Viral

Diarrhea Virus (BDVD) comprising a pharmaceutically effective amount of a virus described above and a carrier, said carrier comprising any physiological buffered medium, i.e. about pH 7.0 to 7.4 containing from about 2.5 to 15% serum which does not contain antibodies to BHV.

A method of immunizing an animal against infectious disease caused by Bovine Viral Diarrhea Virus (BDVD) comprising administering to an animal a pharmaceutically effective amount of a virus or vaccine described herein.

A process of preparing a virus described herein comprising: a) isolation of a functionally expressing gene or gene combination that causes BVDV, b) inserting said gene or gene combination into a replicating nonpathogenic virus, c) selecting a live-virus that functionally expresses the product of said gene or gene combination.

A method of preparing a virus described herein where the functionally expressing gene or gene combination, used to create the virus, is produced by a process comprising the recombination of a plasmid with intact viral DNA, said plasmid comprising: a) a BHV-1 genomic DNA fragment containing the thymidine kinase (tk) gene and having a deletion to the thymidine kinase (tk) gene, b) inserting into the thymidine kinase (tk) gene deletion of said plasmid a promoter/polyadenylation signal, c) inserting a gp53 gene or gene combination between the promoter and the polyadenylation signal, d) transfecting cells with said plasmid to produce a recombinate virus containing said functional gene or gene combination inserted into a live virus that does not cause immunosuppression in the usual host and expressing said functional gene or gene combination.

A method of preparing a virus described herein where the functionally expressing gene or gene combination, used to create the virus, is produced by a process comprising the recombination of a plasmid with intact viral DNA, said plasmid comprising: a) a BHV-1 genomic DNA fragment containing the thymidine kinase (tk) gene and having a deletion to the thymidine kinase (tk) gene, b) inserting into the thymidine kinase (tk) gene deletion of said plasmid a promoter/polyadenylation signal, c) inserting a gp53 gene or gene combination preceded by a signal peptide gene sequence between the promoter and the polyadenylation signal, d) transfecting cells with said plasmid to produce a recombinate virus containing said functional gene or gene combination inserted into a live virus that does not cause immunosuppression in the usual host and expressing said functional gene or gene combination.

BRIEF DESCRIPTION OF THE DRAWINGS.

Figure 1. Construction of the shuttle vectors for inserting foreign genes into BHV-1.

Figure 2. Strategy for appending signal peptide sequences to the BVDV gp53 gene.

Figure 3. Maps of the five shuttle plasmids for inserting gp53 into BHV-1

a. EXAMPLE 1. pBHVtkex-3::p53.

b. EXAMPLE 2: pBHVtkex-1::BGH/p53

c. EXAMPLE 3: pBHVtkex-1::gIII/p53

d. EXAMPLE 4: pBHVtkex-3::BGH/p53

e. EXAMPLE 5:pBHVtkex-3::gIII/p53

Figure 4. Predicted transcript maps of the BHV-1/gp53 recombinant viruses.

Figure 5. Northern blots showing transcription of gp53 messenger RNAs in the BHV-1 recombinants.

Figure 6. Immunoprecipitations showing expression of gp53 protein in the BHV-1 recombinants.

DESCRIPTION OF THE PREFERRED EMBODIMENTS.

All of the terms used below will be readily understood by one skilled in the art. In many places the name of the manufacturer of equipment or reagents are provided in parenthesis after the equipment or reagent is named. Commonly used terms, reagents and buffers such as "plasmids," "Klenow Fragments," "religating blunt ends," "Tris," chelating buffers such as EDTA and EGTA, and commonly used chromatography columns are referred to without further explanation.

In the descriptions of the construction of the compounds used in this invention, standard molecular biological techniques were used and are briefly named or described here. Detailed explanations of these techniques can be found in standard laboratory manuals such as "Molecular Cloning: a Laboratory Manual" (1989), Sambrook, *et. al.*, Cold Spring Harbor Press, Cold Spring Harbor, New York, or "Current Protocols in Molecular Biology" (1991), Ausubel, F. M., *et. al.*, eds., Wiley Interscience, New York.

This invention combines the effectiveness of the attenuated live vaccines with the safety of the inactivated vaccines. We take the gene which codes for gp53, a major glycoprotein of the BVDV virus and against which the host produces substantial immune responses, and express it in bovine herpesvirus -1 (BHV), this recombinant virus (BHV/BVDVgp53) is used as a vaccine against BVDV.

Bovine herpesvirus (BHV) is another major pathogen of cattle which produces respiratory disease. Thus, in common with BVDV, BHV also replicates at a mucosal

surface. With BVDV, replication is mainly at the gut mucosal interface with less replication at the respiratory interface. With BHV it is the respiratory interface which dominates. The common mucosal immune system ensures that immune responses produced at one surface will be effective at other surfaces. Thus the recombinant virus of this invention, BHV/BVDVgp53, will, when administered to cows, sheep or goats, preferably via the intranasal route, replicate in the respiratory mucosae and produce an immune response.

Prior to the expression of the BVDVgp53 gene in BHV, the thymidine kinase gene was deleted from the BHV virus using a process known to attenuate the virus. The BHV, a live attenuated virus, will replicate and produce a cell mediated response. As part of that replicative process, the BVDV gp53 gene will be expressed and, because the virus is inside the cell, the correct processing for a cell mediated response to the BVDV gp53 part of the recombinant virus will also occur. Most importantly, this response will occur without the possible side effects of immunosuppression, as only part of the BVDV virus is present. Thus, the invention combines the efficacy of an attenuated live virus vaccine for BVDV, with the safety of an inactivated preparation.

The examples in the procedures section are provided for illustrative purposes and are in no way intended to limit the scope of the present invention. All media and buffer solutions were made up in glass distilled water unless otherwise indicated.

<u>Compositions and Administrations</u> - A pharmaceutically effective amount of the vaccine of the present invention can be employed along with a pharmaceutically acceptable carrier or diluent as a vaccine against BHV-1 and BVDV in animals, such as bovine, sheep and goats.

Examples of pharmaceutically acceptable carriers or diluents useful in the present invention include any physiological buffered medium, i.e., about pH 7.0 to 7.4, containing from about 2.5 to 15% serum which does not contain antibodies to BHV, i.e., is seronegative for BHV. Serum which does not contain gamma globulin is preferred to serum which contains gamma globulin. Examples of serum to be employed in the present invention include fetal calf serum, lamb serum, horse serum, swine serum, and goat serum. Serum protein such as porcine albumin or bovine serum albumin (hereinafter "BSA") in an amount of from about 0.5 to 3.0% can be employed as a substitute for the serum. However, it is desirable to avoid the use of foreign proteins in the carrier or diluent which will induce allergic responses

in the animal being vaccinated.

The virus may be diluted in any of the conventional stabilizing solutions containing phosphate buffer, glutamate, casitone, and sucrose or sorbose, or containing phosphate buffer, lactose, dextran and glutamate.

It is preferred that the vaccine viruses of the present invention be stored at a titer of at least 10^5 to 10^6 PFU/ml at -70°C to -90°C or in a lyophilized state at 2°C to 7°C. The lyophilized virus may be reconstituted for use with sterile distilled water or using an aqueous diluent containing preservatives such as gentamicin and amphotericin B or penicillin and streptomycin.

The useful dosage to be administered will vary depending upon the age, weight and species of the animal vaccinated and the mode of administration. A suitable dosage can be, for example, about $10^{4.5}$ to 10^7 PFU/animal, preferably about $10^{4.5}$ to $10^{5.5}$ PFU.

The vaccines of the present invention can be administered intranasally, intravaginally or intramuscularly. Intranasally is the preferred mode of administration.

Utility of the Invention - This invention is intended to provide the user with an effective vaccine for prevention of BVDV caused disease, where the vaccine can be safely and efficaciously administered intramuscularly, intranasally, or intravaginally. Intranasally may be the preferred route of administration.

The vaccines of this invention are created with the intention of treating disease, preferably through prevention. By prevent or prevention applicant means to keep the host from developing symptoms of the disease or to mitigate the effects of the disease, that is to avert the typical diseased state. Prevention implies decisive action to stop, impede or delay the onset of disease. Prevention can include the following concepts: to hinder, frustrate, to obstruct; to intercept, possibly prohibit, impede or preclude. Preclude would suggest the onset of the disease state either does not occur or the disease pathogen is largely ineffectual in causing the disease state. Prevent or prevention can indicate the disease state is forstalled, meaning that anticipatory action to prevent or hinder the disease has occurred but the conditions creating the disease have not been eliminated.

The usefulness of this invention will be illustrated by the ability of the vaccine to provide effective protection against the spread of BVDV disease in its various manifestations. Because the vaccine uses gp53, a major glycoprotein of BVDV, and one against which the host produces a substantial immune response, the

vaccine will confer substantial benefits upon the treated potential host. Another object of the invention is to provide a BVDV vaccine which can be administered safely to calves and to pregnant cows in all stages of pregnancy.

Measures of Activity - The vaccine uses gp53, a major glycoprotein of BVDV, and one against which the host should produces a substantial immune response. Others have shown that gp53 is highly immunogenic. Donis, R.O. and Dubovi, E.J., Glycoproteins of Bovine Viral Diarrhoea-Mucosal Disease Virus in Infected Bovine Cells, *Journal of General Virology*, Vol. 68, pp. 1607-1616 (1987). It is well known that agents that produce substantial immune responses can make effective vaccines. Magar, R., *et al.*, Bovine Viral Diarrhea Virus Proteins: Heterogeneity of Cytopathogenic and Noncytopathogenic Strains and Evidence of 53K Glycoprotein Neutralization Epitope, *Veterinary Microbiology*, Vol. 16, pp. 303-314. The vaccines of this invention contain genes that express large quantities of gp53, this is shown in figure 5. Because of the expression of large quantities of gp53 the vaccines of this invention will confer substantial benefits upon the treated potential host.

Preferred Compounds - Any BHV-1 virus attenuated with a tk deletion and carrying the gp53 gene, the gp53 gene being preceded by a signal peptide, that expresses abundant amounts of gp53, should be a preferred suitable vaccine candidate. It appears the signal peptide sequence may be taken from any suitable source. We chose to examine two different signal peptides to ensure the best localization of the gp53 protein *in vivo*. We chose two candidates we call "T11-6", embodied in Example 2, and "T11-3", embodied in Example 3 for vaccine trials. The former virus was deposited to the ATCC under the designation UC VR-58. The latter, "T11-3" plasmid was also deposited. The virus we labeled "T11-8" might contain truncated forms of the tk transcript and this might suggest, but does not necessarly mean, that it would be less attractive as a vaccine candidate. A large number of existing cell lines are persistently infected with non-cytopathic BVDV from passage in media containing fetal bovine serum taken from infected calves. For this invention, it is imperative that viruses used as live, attenuated vaccines are free of contaminating BVDV.

Preparation of the Compounds

Construction of expression shuttle vectors for gene insertion into Bovine herpesvirus type-1 (BHV-1).

We constructed two shuttle vectors to allow insertion of foreign genes into BHV-1. Although this invention shows the utility of BHV-1 as a vector for BVDV

genes many other viruses could fill the same role. Other examples from cattle, sheep and goats would include cow, goat and sheep pox viruses, adenoviruses, bovine mammillitis virus, bovine papillomavirus, and pseudorabies virus. A non-pathogenic virus refers to any virus which has the ability to replicate in one of its host species but does not produce any signs of disease in that species. Such non-pathogenic viruses might arise from pathogenic parent viruses by natural mutation, might be mutagenized by, for instance, chemicals or light to produce a non-pathogenic virus, or could be rendered non-pathogenic through the use of recombinant DNA technologies. See, 1) Mapping Neutralization Domains of Viruses, E.Wimmer, E.A. Emini, and D.C. Diamondand 2) Immunogenicity of Vaccine Products and Neutralizing Antibodies, E Norrby. Both articles are in Edited by Notkins and Oldstone Published by Springer-Verlag New York Inc. 1986.

Since we intended to attenuate BHV-1 by inactivating the viral thymidine kinase (tk)(M. Kit, et al., US Patent 4,703,011, (1983)), we decided to use the BHV-1 tk gene for the site of insertion. This approach not only insured the complete inactivation of the viral tk, but also allowed us to select recombinant, tk-negative virus by established methods. M. F. Shih, et al., *Proc Natl Acad Sci USA*, 81:5867-5870 (1984). Other methods to attenuate BHV-1, such as deletion of other non-essential genes would also be applicable to this particular invention. We started with plasmid pHAS4 which contains a 2.7kb SalI subfragment of the BHV-1 HindIII-A fragment cloned into plasmid pUC18. E. Petrovkis, unpublished data. M. Engels, et al., *Virus Res*, 6:57-73 (1986); J. E. Mayfield, et al., *J Virol*, 47:259-264 (1983); A. L. Meyer, et al., *Biochim Biophys Acta*, 1090:267-9 (1991). As shown in Fig. 1, this SalI fragment contained the entire tk gene, as well as a portion of the upstream gene homologous to the HSV-1 UL24 gene, and a portion of the glycoprotein H gene. L. J. Bello, et al., *Virology*, 189:407-414 (1992); J. G. Jacobson, et al., *J Virol*, 63:1839-1843 (1989); M. Kit, et al., US Patent 4,703,011, (1983); A. L. Meyer, et al., *Biochim Biophys Acta*, 1090:267-9 (1991).

A 424bp deletion was introduced into the tk gene by digesting pHAS4 with BglII and XhoI, filling in the ends with the Klenow Fragment of DNA polymerase I (Klenow) and religating the resulting blunt ended fragments. This manipulation restored the BglII recognition site, but not the XhoI site (Fig. 1). The resulting plasmid was named pHAS4ΔBX. This deletion was chosen because it does not impede on the previously identifed transcription initiation sites for the UL24 homolog which overlaps the 5' end of the tk gene. L. J. Bello, et al., *Virology*,

189:407-414 (1992); J. G. Jacobson, et al., *J Virol*, 63:1839-1843 (1989). Numerous other deletions within the BHV-1 tk gene would be possible. To facilitate later cloning manipulations, we eliminated the HindIII site in the pUC18 vector by digesting pHAS4ΔBX with HindIII, filling in the cohesive ends with Klenow, and religating the blunt ends.

We obtained a 1775bp cassette containing the Human cytomegalovirus (CMV) major immediate early promoter and the bovine growth hormone polyadenylation sequence. R. J. Brideau, et al., *J Gen Virol*, 74:471-477 (1993). These gene expression signals are commonly used for high levels of expression of foreign genes in a number of different systems, but other promoter/polyadenylation signal pairs could also be used in this context. The cassette, in vector p3CL-DHFR, is bounded by unique EcoRI and BglII sites and contains, between the promoter and the polyadenylation signal, unique HindIII and SalI restriction sites for cloning of foreign genes. The p3CL-DHFR vector was digested with EcoRI, then filled in and ligated to a BamHI linker (New England Biolabs, Beverly, Massachusetts). This manipulation regenerated the EcoRI site. The construct was then digested with BamHI and BglII and the released cassette was ligated into the BglII site of pHAS4ΔBX (Fig. 1). The ligations were transformed into *E. coli* strain DH5α. We isolated recombinant plasmids that contained the p3CL insert in both orientations relative to the BHV-1 tk gene by mapping of asymmetric restriction sites. These two constructs, designated pHAS4ΔBXex-1 and pHAS4ΔBXex-3 (Fig. 1), contained then, a strong promoter and polyadenylation signal bounded by the BHV-1 tk gene and flanking regions to allow homologous recombination into the BHV-1 genome.

Figure 1. Construction of shuttle vectors for inserting foreign genes into BHV-1. PHAS4 is a 2.7kb subfragment from the BHV-1 HindIII-A fragment. The BglII/XhoI subfragment to be deleted is shown. The deletion derivative of pHAS4 is pHAS4ΔBX. The deleted thymidine kinase (tk) gene is shown as a dark stippled box. The cassette containing the promoter and polyadenylation signal is shown just below pHAS4ΔBX. The CMV immediate early promoter is shown as a light stippled box, and the Bovine Growth Hormone (BGH) polyadenylation signal is shown as a striped box. Finally, the inserts of the two expression shuttle plasmids, pHAS4ΔBXex1 and pHAS4ΔBXex3 are shown.

Addition of Signal Peptide Sequences to BVDV gp53 gene.

A cDNA containing the BVDV gp53 gene from strain 2724, a noncytopathic strain, has been previously described. Kennedy, M. et al, abstracts of the American

College of Veterinary Microbiologists, 1992 workshop. Since the BVDV RNA genome is normally translated as one long polyprotein and then post-translationally modified into the various viral proteins, the gp53 portion of the BVDV genome does not contain the usual signal peptide required for translocation of the protein to the cell membrane, where the protein is normally expressed. Nonetheless, the cDNA was successfully expressed in both cell-free systems and baculovirus, and the protein appeared to be translocated, glycosylated and anchored in both systems, despite the lack of a conventional signal peptide. We decided, however, to evaluate expression of gp53 in BHV-1 both with and without various signal peptides.

In order to attach nucleotide sequences encoding signal peptides to the gp53 gene, we introduced a BamHI site into 5' end of the p53 gene by site directed mutagenesis, as follows: The p53 gene was blunt-end ligated into the filled-in BamHI site of plasmid pSP72 (Promega Corp., Madison, Wisconsin), thus removing all BamHI sites from the resulting plasmid. We introduced a single base change, a C to a G, 11 bases in from the initiation codon used by the cDNA, using a synthetic oligonucleotide and the "Double Take" site directed mutagenesis kit (Stratagene, La Jolla CA) according to the manufacturer's instructions. This base change introduced a unique BamHI site into the gene without altering the gp53 amino acid sequence (Fig. 2 section B). The base change was verified by nucleotide sequencing, and the resulting plasmid was called pP53mut. We inserted, into pP53mut sequences, encoding signal peptides from the PRV gIII gene (A. K. Robbins, et al., *J Virol*, 58:339-347 (1986)) and from Bovine growth hormone. R. P. Woychik, et al., *Nucl Acids Res*, 10:7197-7210 (1982). (Figure 2 section A) Complentary oligonucleotides encoding the two signal peptides were synthesized such that annealed oligos had SalI cohesive ends 5' and BamHI cohesive ends 3' (Fig 2 section A). These signal peptide cassettes were ligated into pP53mut digested with BamHI and SalI, and transformed into DH5α. We confirmed the correct insertion of the signal peptide cassettes by nucleotide sequencing.

Complementary oligonucleotides encoding any well characterized signal peptide can be used in this invention. Thirty-nine examples of well characterized signal peptide sequences found in Perlman, D., et al., *J. Mol . Biol.* Vol. 167 pp. 391-409 (1983). Incorporated by reference. These and any other well characterized signal peptides should be suitable for use as embodiments of this invention.

Figure 2. Strategy for appending signal peptide sequences to the BVDV gp53 gene. Section A: Synthetic oligonucleotides corresponding to the signal

peptide sequences of Bovine Growth Hormone (BGH), and Pseudorabies virus gIII (PRV gIII). Complementary oligonucleotides were synthesized such that the annealed pairs had SalIsites on the 5' ends and BamHI sites on the 3' ends. The deduced amino acid sequences of the signal peptides are also shown. In each case the predicted cleavage sites for the signal peptides are just after the alanine (A), three amino acids from the ends. Codons for two amino acid residues (F,P in BGH; P,S in gIII) from the original native proteins were left on the signal peptide sequences to ensure correct cleavage.

Section **B**: Site directed Mutagenesis of the cDNA encoding the BVDV gp53 gene. The first 60 nucleotides of the gp53 cDNA and the corresponding amino acid sequence are shown. A single base pair, shown by the arrow, was changed to create a BamHI restriction site in the sequence, shown in the box. This change does not change the amino acid sequence. The cDNA was then digested with BamHI as shown, allowing in frame ligation to either of the signal peptide sequences shown in section A.

Other expression gene fragments in addition to gp53.

Expression of other BVDV gene or gene combinations in a live virus vector are also embodiments of this invention. This would include any and all BVDV proteins to which a vaccinated animal could elicit an immune response. Examples include, but are not limited to, the other two BVDV surface glycoproteins, gp48 and gp25 (Collett, M.S., et al., *Virology* 165:200-208 (1988)), the p14 capsid protein (Thiel, H.J., et al., *J. Virol.* 65:4705-4712 (1991)), and the p20 N-terminal protease. Wiskerchen, M., et al., J. Virol. 65:4508-4514 (1991). This group of proteins, along with the gp53 gene, can be expressed together from a single cDNA molecule, the expressed polyprotein will process itself correctly into the separate proteins. Another BVDV protein candidate to express in a vaccine is the nonstructural p125/p80 protein (Deregt, D., et al., *Can. J. Microbiol.* 37:815-122 (1991)), which elicits a significant antibody response in infected cows.

Insertion of the BVDV gp53 gene into the BHV-1 expression vectors.

The p53 gene, either with or without added signal peptide sequences, was ligated into the HindIII insertion sites of pHAS4ΔBXex-1 and pHAS4ΔBXex-3 by filling in all the respective cohesive ends of vectors and inserts followed by blunt end ligation. The ligations were transformed in *E. coli* strain DH5α. We wanted to eventually evaluate the expression of gp53 in BHV-1 in various orientations and with at least two different signal peptides to ensure that we achieved the most

efficient expression. The transformed colonies were screened by colony hybridization using as a probe the p53 insert labelled with Digoxygenin-dUTP. The "Genius" DNA hybridization system (Boeringer Mannheim Biochemicals (BMB), Indianapolis, IN) was used for this and all other DNA hybridizations described in the characterization of this invention. Positive recombinants were then screened by restriction analysis for those carrying the gp53 gene in the proper orientation relative to the CMV promoter and BgH polyadenylation signal. Five plasmids were isolated, which are schematically depicted in Figs. 3A-E. Their descriptions are as follows.

EXAMPLE 1. pBHVtkex-3::p53: contains the BVDV gp53 gene inserted between the CMV promoter and the BGH polyadenylation signal of pHAS4ΔBXex-3 with no added signal peptide. In this construct the original gp53 gene, PRIOR to site directed mutagenesis, was inserted. See Fig. 3A. This plasmid was then used to construct the virus T2-3#.

EXAMPLE 2. pBHVtkex-1::BGH/p53: contains the mutagenized gp53 gene preceded by the BGH signal peptide sequence inserted into pHAS4ΔBXex-1. See Fig. 3B. This plasmid was used to create the virus T11-6. This virus was deposited.

EXAMPLE 3. pBHVtkex-1::gIII/p53: contains the mutagenized gp53 gene preceded by the PRV gIII signal peptide sequence inserted into pHAS4ΔBXex-1. See Fig. 3C. This plasmid was used the create the virus T11-3. This plasmid was deposited.

EXAMPLE 4. pBHVtkex-3::BGH/p53: contains the mutagenized gp53 gene preceded by the BGH signal peptide sequence inserted into pHAS4ΔBXex-3. See Fig. 3D.

EXAMPLE 5. pBHVtkex-3::gIII/p53: contains the mutagenized gp53 gene preceded by the PRV gIII signal peptide sequence inserted into pHAS4ΔBXex-3. See Fig. 3E. This plasmid was used to create the virus T11-8. This plasmid was deposited.

Figures 3A-E. Complete maps of the five shuttle plamids for inserting gp53 into BHV-1. The gp53 gene is shown as a solid band, the BHV-1 sequences are shown as dark stippled bands, the CMV promoter region is shown as a light stippled band, and the BGH polyadenylation signal region is shown as a striped band. The plasmid vector, pUC18, is shown as a thin line. In each case the direction of transcription of gp53 relative to the original direction of transcription of BHV-1 tk is shown. The various signal peptide sequences are indicated.

a. EXAMPLE 1. pBHVtkex-3::p53.

b. EXAMPLE 2. pBHVtkex-1::BGH/p53

c. EXAMPLE 3. pBHVtkex-1::gIII/p53

d. EXAMPLE 4. pBHVtkex-3::BGH/p53

e. EXAMPLE 5. pBHVtkex-3::gIII/p53

These, and all other possible insertions of the BVDV gp53 gene into the BHV-1 tk gene are embodiments of this invention. These plasmids and any plasmids created in this manner are known as "Principal Plasmid Vectors" and are the plasmid vectors used to create the virus vaccines of this invention.

Introduction of the gp53 gene into BHV-1 "Iowa".

The five expression shuttle plasmids carrying gp53 were linearized by XbaI and cotransfected into Bovine Turbinate (BT) cells with unit length DNA from BHV-1 strain Iowa (tk positive) by the standard $CaPO_4$ method (R. L. Graham, et al., *Virology*, 52:456-467 (1973)) as modified by Cai (W. Cai, et al., *J Virol*, 61:714-721 (1987)) . The cells were obtained from ATCC. The transfections were then subjected to two rounds of selection either on 143tk$^-$ cells (S. K. Mittal, et al., *J Gen Virol*, 70:(1989)), or on Rab (BU) cells (S. Kit, et al., *Virology*, 130:381-389 (1983)) in the presence of 100ug/ml 5-Bromo-2'-Deoxyuridine (BDUR, Sigma Chemical Company, St. Louis, Missouri) to isolate virus no longer expressing tk. This is a standard procedure described previously. M. Kit, et al., US Patent 4,703,011, (1983). Other tk$^-$ cell lines permissive for growth of BHV-1 can also be used. After the two rounds of BDUR passage, transfections that still showed cytopathic effect were infected onto BT cells under complete media with 1% low melting agarose to obtain single plaques. Multiple single plaques were picked from each transfection and the viral DNAs were screened for the p53 gene by dot-blot DNA hybridization. Although not all transfections survived the BDUR passages (particularly those on the 143 tk$^-$ cells, as these cells are only marginally permissive for BHV-1 viral growth), those that did survive yielded 100% recombinant virus. Four different recombinant viruses were isolated and further characterized:

EXAMPLE 1. T2-3#3 and T2-2#5 (two identical, but independently isolated viral clones): BHV-1 "Iowa" into which the insert sequences contained in pBHVtkex-3::p53 recombined. Contains the BVDV gp53 gene with no added signal peptide sequence situated between the CMV promoter and the BGH polyadenlyation signal, with transcriptional orientation in the same direction as the BHV-1tk gene.

EXAMPLE 2. T11-6 (This virus was submitted to ATCC under the designation UC VR-58): BHV-1 "Iowa" into which the insert sequences contained in pBHVtkex-1::BGH/p53 recombined. Contains the BVDV gp53 gene with the BGH signal peptide sequence situated between the CMV promoter and the BGH polyadenlyation signal, with transcriptional orientation in the opposite direction relative to the BHV-1 tk gene.

EXAMPLE 3. T11-3: BHV-1 "Iowa" into which the insert sequences contained in pBHVtkex-1::gIII/p53 recombined. Contains the BVDV gp53 gene with the PRV gIII signal peptide sequence situated between the CMV promoter and the BGH polyadenlyation signal, with transcriptional orientation in the opposite direction relative to the BHV-1 tk gene.

EXAMPLE 5. T11-8: BHV-1 "Iowa" into which the insert sequences contained in pBHVtkex-3::gIII/p53 recombined. Contains the BVDV gp53 gene with the PRV gIII signal peptide sequence situated between the CMV promoter and the BGH polyadenlyation signal, with transcriptional orientation in the same direction as the BHV-1 tk gene.

A virus was not isolated from cotransfections with "Iowa" DNA and plasmid pBHVtkex3::BGH/p53, **EXAMPLE 4**, but this prophetic virus, could be easily created, it and any other BHV-1 viruses containing the BVDVgp53 gene inserted into thymidine kinase gene are embodiments of this invention. We purified DNA from each of these viruses and checked for the proper insertions in the proper orientations by Southern Hybridization using both the gp53 gene and the CMV promoter/BgH polyadenylation cassette as probes (data not shown). All four of the viruses carried the complete promoter/gene/polyadenylation cassettes in the BHV-1 tk gene, deleted as predicted, based on restriction fragment sizes. As a control, with these transfections, we also transfected the pHAS4ΔBX plasmid with BHV-1 "Iowa" unit length DNA and isolated a tk-negative progeny carrying the 424bp deletion in tk (also verified by Southern Hybridization). This virus is named IowaΔBX. All of these viruses were plaque purified twice by limiting dilution on BT cells.

A large number of existing cell lines are persistently infected with non-cytopathic BVDV from passage in media containing fetal bovine serum taken from infected calves. For this invention, it is imperative that viruses used as live, attenuated vaccines are free of contaminating BVDV. In order to ensure that the BHV-1 viruses carrying the BVDV sequences were not contaminated with non-cytopathic BVD virus, we prepared DNA from each of the viruses (including the

parent strain Iowa and IowaΔBX) and subjected the DNA preps to extensive RNAse treatment using a cloned RNAse (RNAse ONE, Promega Corporation, Madison, Wisconsin). Since BVDV has only RNA as its genetic material, this manipulation should eliminate any possible contaminating BVDV sequences from the viral DNA preps. We then transfected these RNAsed viral DNAs into certified BVD-free MDBK cells (ATCC) and picked virus plaques from the transfections to use in further manipulations.

Transcriptional analysis of the gp53 recombinants.

We prepared RNA from each of the recombinant viruses and the parent BHV-1 strain Iowa and evaluated transcription of gp53 by Northern hybridization. A diagram of the possible message species and the probes used is shown in Fig. 4.

Figure 4. Predicted transcripts of the BHV-1/gp53 recombinant viruses later shown in Figure. 5. The two probes are 1) the gp53 cDNA and 2) the SalI/BglII portion of pHAS4 (shown above the maps). The first map shows the predicted transcripts from viruses T11-3 and T11-6, and the second map shows the predicted transcripts from T11-8. The sites of transcript initiation for tk and UL24 are shown for reference.

All of the gp53 recombinant viruses made a 1.6kb message that hybridized with a ^{32}P-labelled gp53 probe, the size predicted for transcription initiation at the CMV promoter and termination at the BgH polyadenylation site , Fig. 5, probe 1. The T2-3#3 and T2-2#5 virus are not shown. As additional major bands, T11-3 and T11-6 made an 8.5kb transcript and T11-8 and T2-3#3 made a 5.6kb transcript. These transcripts were unique to the recombinant viruses, and were consistent with messages initiating at the CMV promoter, reading through the BgH poly adenylation signal and terminating at the UL24 or tk/gH polyadenylation signals, respectively.

Hybridization with the upstream and downstream probes confirmed the identity of these longer messages. The p53 probe did not hybridize to Iowa, IaΔBX or mock infected RNAs. As a quantitation control we used probe pHAS6, an 867bp salI fragment that maps downstream of the tk open reading frame and is internal to the gH gene. A. L. Meyer, et al., *Biochim Biophys Acta*, 1090:267-9 (1991). All of the viruses made equivalent amounts of the 3.1kb gH message (data not shown). This probe also hybridized to the longer p53 messages in T11-8 and T2-3-3, and to the 4.3kb tk message in Iowa, which is 3' coterminal with the gH transcript. L. J. Bello, et al., *Virology*, 189:407-414 (1992).

To examine the transcription patterns upstream of the gp53 insertions, we

used a probe that consisted of the pHAS4 fragment from the upstream SalI site to the BglII site in the tk gene, the beginning of the deletion in the recombinant viruses (probe 2). All of the viruses made a message of approximately 4.4kb which we deduced to be UL24 (Fig. 5, probe 2). This message, however, was smaller than the 5.2kb UL24 message in BHV-1 strain Cooper described by Bello, et al (L. J. Bello, et al., *Virology*, 189:407-414 (1992)) and comigrated with the tk message in the wild-type strain Iowa. Although we did not evaluate these comigrating messages further by using single stranded probes, we detected a tk transcript of 4.2 kb only in the Iowa DNA with probe pHAS6 and we detected similarly sized transcripts in all the viral RNAs with the upstream probe, even though these other viruses cannot be making a wild-type sized tk transcript. In T11-3 and T11-6, the upstream probe did not detect any truncated forms of tk message and hybridzed to only the UL24 message and the the 8.5kb p53 message. In T11-8, on the other hand, the probe hybridized to four additional (minor) bands of approximately 5.0, 3.7, 1.8, and 1.0kb.

Figure 5. Northern blots showing transcription of gp53 messenger RNAs in the BHV-1 recombinant viruses. The first panel shows transcripts hybridizing to probe 1, the pg53 cDNA, and the second panel shows transcripts hybridizing to probe 2, the SalI/BglI subfragment of pHAS4. KEY: M=Mock infected cells, I=BHV-1 "Iowa" infected cells, 3,6,8=T11-3, T11-6 and T11-8 infected cells. RNA size standards, in kilobases (kB) are given to the left of each panel.

Expression of BVDV gp53 protein in BHV-1.

We evaluated expression of gp53 protein in the BHV-1 recombinants by immunoprecipitation (IP). Detailed procedures for IPs can be found in standard references such as "Current Protocols in Molecular Biology", Ausubel, F. M., et. al., eds., Wiley Interscience, New York. BT cells infected with the BHV-1 recombinants were metabolically labelled with ^{35}S-methionine (Amersham, Arlington Heights, Illinois). The viral infected cells were lysed and soluble proteins were reacted with hyperimmune serum from bovine or goat against BVDV. VMRD, Pullman, Washington. Antigen/antibody complexes were precipitated staph A (Immunoprecipitin, Gibco/BRL, Gaithersburg, Maryland,) or protein A sepharose 4B (Pharmacia, Uppsala, Sweden). Immunoreactive proteins were resolved by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

Figure 6 shows that all three of the recombinant viruses carrying the gp53 gene preceded by a signal peptide sequence made significant amounts of the protein.

We did not detect any expression of gp53 from T2-3#3, or T2-2#5 the viruses carrying the gp53 gene, but lacking a signal peptide, even though this virus synthesized considerable amounts of gp53 messenger RNA. The clones t2-3#3 and T2-2#5 are independently isolated clones, which rules out the possibility that one particular virus had a defect that precluded gp53 expression (data not shown). The possiblity remains that gp53 is being synthesized from T2-3, but is rapidly degraded, or that our antibody does not detect unprocessed forms of the protein.

Figure 6. Immunoprecipitated proteins showing expression of gp53 in the BHV-1 recombinants. Labelled proteins were precipitated with polyclonal bovine-anti-BVDV serum, this serum also had minor reactivity with BHV-1 antigens. KEY: 3,6,8=T11-3, T11-6, and T11-8 infected cell proteins, IA=BHV-1 "Iowa" infected cell proteins, M=Mock infected cell proteins. MW=approximate protein molecular weight standards, in Kilodaltons.

The gp53 protein bands in T11-3, T11-6 and T11-8 were broad, suggesting that the proteins were processed, and they appeared to be equivalent but not identical in size to the gp53 protein in NADL (data not shown). Removal of the N-linked sugars from the BVDV-NADL and BHV-1 expressed gp53 proteins by digestion with N-glycansase (Genzyme, Cambridge, Massachusetts) did not resolve the size difference in the proteins, but the proportional reduction in size of the proteins suggested that the native and recombinant forms of gp53 were processed similarly. The slight size difference between the recombinant and native proteins could be due to the fact that the gp53 gene in the BHV-1 viruses came from a different BVD strain which could have a gp53 of a slightly different size, or the cDNA gp53 clone might not contain the exact amino acids processed from the BVDV polyprotein into native gp53.

The present invention is not to be limited in scope by the cell lines deposited or the embodiments disclosed herein which are intended as single illustrations of one aspect of the invention and any which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair and amino acid residue numbers and sizes given for nucleotides and peptides are approximate and used for the purposes of description.

All documents cited herein are incorporated by reference.

Deposit of Genetic Materials

One skilled in the art should be able to reconstruct all the various embodiments of this invention by utilizing only the written description. However, for the sake of completeness, to ensure enablement, and to provide every opportunity for others to make and use this invention, certain genetic constructs of this invention have been deposited at recognized depositories in accordance with the Budapest Treaty.

A virus was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, zip code 20852, USA. That deposit was designated UC VR-58 by the Upjohn Company and given the following number by the depository, ATCC No. VR2436, it corresponds to the virus described herein as "T11-6," also known as "Example 2." This deposit was received by the American Type Culture Collection depository on 28 October 1993.

Several plasmids were deposited with the Agricultural Research Service Culture Collection (NRRL), of the U.S. Department of Agriculture, at 1815 North University Street, Peoria, Illinois, zip code 61604, USA. One plasmid was given the Upjohn designation, pUC 1564, *E. coli* culture UC 15085, referring to pBHVtkex-1::gIII\p53, it corresponds to the plasmid used to create the virus described herein as "T11-3," also known as "Example 3." This plasmid was given the following number by the depository, NRRL B-21350. Another deposit was given the Upjohn designation, pUC 1565, *E. coli* culture UC 15086, referring to pBHVtkex-3::gIII\p53, it corresponds to the plasmid used to create the virus described herein as, "T-11-8," also known as "Example 5." This plasmid was given the following number by the depository, NRRL B-21351. Both of the plasmids were received by the Agricultural Research Service Culture Collection depository on 26 October 1994.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Upjohn Company
INVENTORS (For U.S. Purposes only): Wardley, Richard C. and Haanes, Elizabeth J.

(ii) TITLE OF INVENTION: A Replicating Nonpathogenic Virus Expressing Envelope Glycoproteins from Bovine Viral Diarrhea Virus (BVDV)

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(D) STATE: Michigan
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(F) ZIP: 49001-0199

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 35,004
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8083 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bovine viral diarrhea virus
(B) STRAIN: 2724
(C) INDIVIDUAL ISOLATE: pBHVtkex-3::p53

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8149 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bovine viral diarrhea virus
(B) STRAIN: 2724
(C) INDIVIDUAL ISOLATE: pBHVtkex-1::gBGH/p53

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8135 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bovine viral diarrhea virus
(B) STRAIN: 2724
(C) INDIVIDUAL ISOLATE: pBHVtkex-1::gIII/p53

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8149 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bovine viral diarrhea virus
(B) STRAIN: 2724
(C) INDIVIDUAL ISOLATE: pBHVtkex-3::BGH/p53

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8135 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bovine viral diarrhea virus
(B) STRAIN: 2724
(C) INDIVIDUAL ISOLATE: pBHVtkex-3::gIII/p53

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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Claims

1. A replicating nonpathogenic virus, for preventing disease caused by Bovine Viral Diarrhea Virus (BVDV), where said replicating nonpathogenic virus comprises:

a gene or gene combination taken from a BVDV virus, and said replicating nonpathogenic virus functionally expresses said gene or gene combination.

2. A virus of claim 1, where said replicating nonpathogenic virus is attenuated.

3. A virus of claim 2, where said replicating nonpathogenic virus is selected from attenuated Bovine Herpes Virus type 1 (BHV-1), attenuated adenoviruses, attenuated bovine mammillitis virus, attenuated bovine papillomavirus, or attenuated pseudorabies virus.

4. A virus of claim 2, where said replicating nonpathogenic virus is attenuated and contains and expresses any combination of the following genes: the genes that code for gp48, gp25, p14 capsid protein, p20 N-terminal protease and p125/p80 protein.

5. A virus of claim 3, where said replicating nonpathogenic virus is attenuated and contains and expresses any combination of the following genes: the genes that code for gp48, gp25, p14 capsid protein, p20 N-terminal protease and p125/p80 protein.

6. A virus of claim 2, where said attenuation is created by making the thymidine kinase (tk) gene nonfunctional.

7. A virus of claim 3, where said attenuation is created by making the thymidine kinase (tk) gene nonfunctional.

8. A virus of claim 4, where said attenuation is created by making the thymidine kinase (tk) gene nonfunctional.

9. A virus of claim 5, where said attenuation is created by making the thymidine kinase (tk) gene nonfunctional.

10. A virus of claim 9, where said replicating nonpathogenic virus is attenuated Bovine Herpes Virus type 1 (BHV-1).

11. A virus of claim 10, where said replicating nonpathogenic virus contains and expresses the gene that codes for gp53, a glycoprotein of the Bovine Viral Diarrhea Virus (BVDV).

12. A virus of claim 11, where a signal peptide is inserted preceeding the gene or gene combination that codes for gp53 in said Bovine Herpes Virus type 1 (BHV-1).

13. A virus of claim 12, where said gene that codes for gp53 is inserted into the inactivated thymidine kinase (tk) gene site.

14. A virus of claim 13, where the functionally expressing gene or gene combination, used to create the virus, comprises a recombined plasmid with intact viral DNA, said plasmid comprising:

a) a BHV-1 genomic DNA fragment containing the thymidine kinase (tk) gene and having a deletion to the thymidine kinase (tk) gene,

b) a promoter/polyadenylation signal inserted in the thymidine kinase (tk) gene deletion,

c) a signal peptide gene sequence preceding a gp53 gene or gene combination all of which is inserted between the promoter and the polyadenylation signal.

15. A virus of claim 14, where said signal peptide gene sequence is taken from any well characterized signal peptide sequences such as any of the thirty-nine examples of well characterized signal peptide sequences found in Perlman, D., et al., *J. Mol . Biol.* Vol. 167 pp. 391-409 (1983).

16. A virus of claim 15, where said signal peptide gene sequence is taken from Psuedorabies Virus gIII gene (PRV) and/or Bovine Growth Hormone (BGH).

17. A virus of claim 16 where the plasmid is selected from the following plasmids,

a) pBHVtkex-1::BGH/p53;

b) pBHVtkex-1::gIII/p53;

c) pBHVtkex-3::BGH/p53; or

d) pBHVtkex-3::gIII/p53.

18. A virus of claim 17, where said virus that produces the product of a functionally expressing gene or gene combination is selected from one of the following viruses,

T11-3, T11-6, or T11-8.

19. A virus of claim 18, where said gene or gene combination is T11-6.

20. A virus of claim 11, where said gene that codes for gp53 is inserted into the inactivated thymidine kinase (tk) gene site.

21. A virus of claim 20, where the functionally expressing gene or gene combination, used to create the virus, comprises a recombined plasmid with intact viral DNA, said plasmid comprising:

a) a BHV-1 genomic DNA fragment containing the thymidine kinase (tk) gene and having a deletion to the thymidine kinase (tk) gene,

b) a promoter/polyadenylation signal inserted in the thymidine kinase (tk) gene deletion,

c) a gp53 gene or gene combination inserted between the promoter and the polyadenylation signal.

22. A virus of claim 21, where said plasmid is made from a plasmid having the characteristics of plasmid pHAS4.

23. A virus of claim 22, where said plasmid is pBHVtkex-3::p53.

24. A virus of claim 23, where said virus is selected from one of the following viruses,

T2-3#3 or T2-2#5.

25. A vaccine for preventing disease caused by Bovine Viral Diarrhea Virus (BDVD) comprising a pharmaceutically effective amount of a virus of claim 1 and a carrier.

26. A vaccine as claimed in claim 25, for preventing disease caused by Bovine Viral Diarrhea Virus (BDVD) comprising a pharmaceutically effective amount of a virus of claim 1 and a carrier, said carrier comprising any physiological buffered medium, i.e. about pH 7.0 to 7.4 containing from about 2.5 to 15% serum which does not contain antibodies to BHV.

27. A method of immunizing an animal against infectious disease caused by Bovine Viral Diarrhea Virus (BDVD) comprising administering to an animal a pharmaceutically effective amount of a virus of claim 1.

28. A process of preparing a virus of claim 1 comprising:

a) isolation of a functionally expressing gene or gene combination that causes BVDV,

b) inserting said gene or gene combination into a replicating nonpathogenic virus,

c) selecting a live-virus that functionally expresses the product of said gene or gene combination.

29. A method of preparing a virus of claim 11 where the functionally expressing gene or gene combination, used to create the virus, is produced by a process comprising the recombination of a plasmid with intact viral DNA, said plasmid comprising:

a) a BHV-1 genomic DNA fragment containing the thymidine kinase (tk) gene and having a deletion to the thymidine kinase (tk) gene,

b) inserting into the thymidine kinase (tk) gene deletion of said plasmid a promoter/polyadenylation signal,

c) inserting a gp53 gene or gene combination between the promoter and the polyadenylation signal,

d) transfecting cells with said plasmid to produce a recombinate virus containing said functional gene or gene combination inserted into a live virus that does not cause immunosuppression in the usual host and expressing said functional gene or gene combination.

30. A method of preparing a virus of claim 12 where the functionally expressing gene or gene combination, used to create the virus, is produced by a process

comprising the recombination of a plasmid with intact viral DNA, said plasmid comprising:

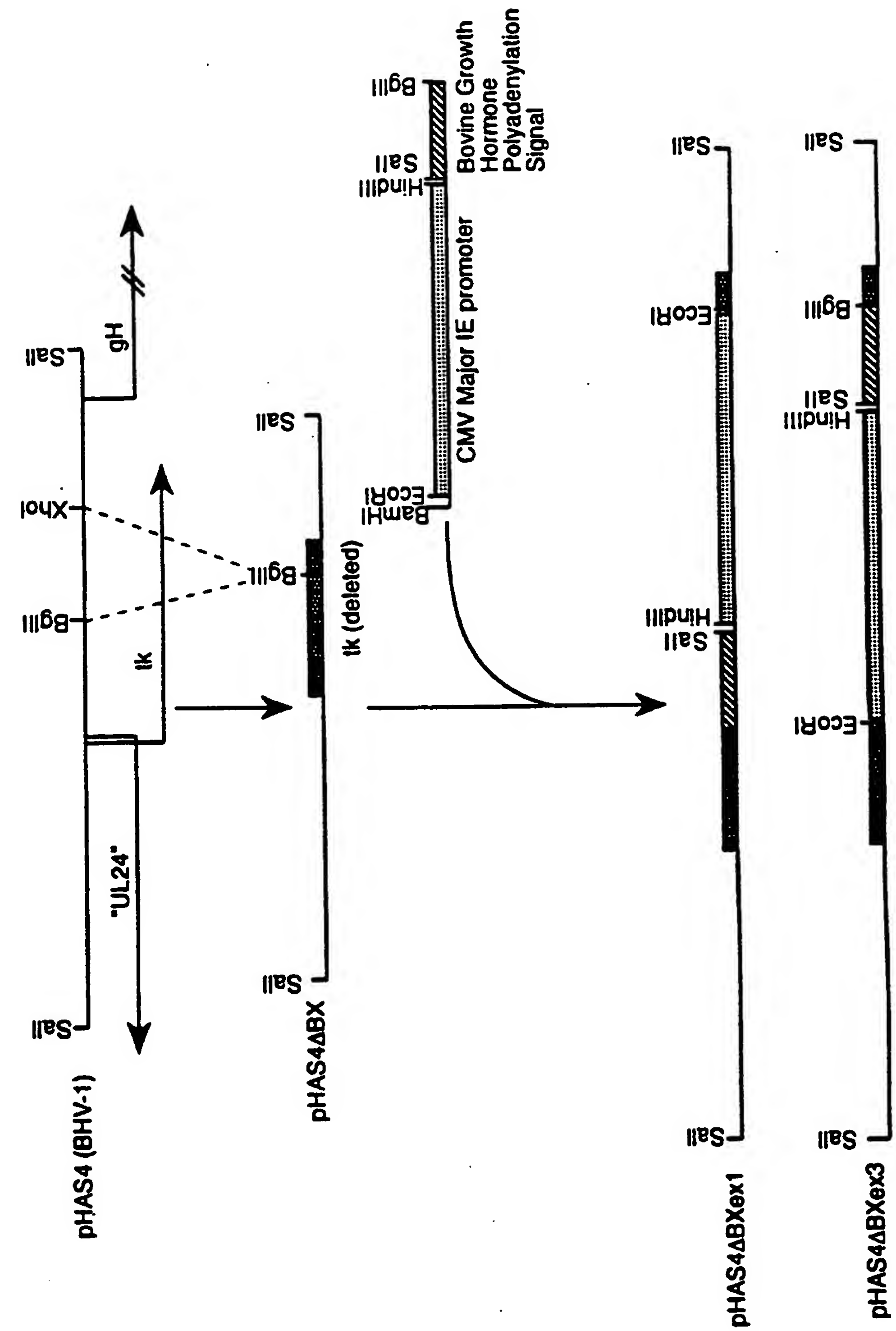
a) a BHV-1 genomic DNA fragment containing the thymidine kinase (tk) gene and having a deletion to the thymidine kinase (tk) gene,

b) inserting into the thymidine kinase (tk) gene deletion of said plasmid a promoter/polyadenylation signal,

c) inserting a gp53 gene or gene combination preceded by a signal peptide gene sequence between the promoter and the polyadenylation signal,

d) transfecting cells with said plasmid to produce a recombinate virus containing said functional gene or gene combination inserted into a live virus that does not cause immunosuppression in the usual host and expressing said functional gene or gene combination.

FIGURE 1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SalI
BglII
XhoI
SalI
gH
tk
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BglII
SalI
tk (deleted)
BamHI
EcoRI
CMV Major IE promoter
HindIII
SalI
BglII
Bovine Growth Hormone Polyadenylation Signal
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SalI
SalI
HindIII
EcoRI
SalI
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HindIII
SalI
BglII
SalI

FIGURE 2A

BGH

```
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---------+---------+---------+---------+---------+---------+
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     M  M  A  A  G  P  T  Y  S  L  L  L  A  F  A  L  L  C

                                         BamHI
CTGCCCTGGACTCAGGTGGTGGGCGCCTTCCCAGGG
---------+---------+---------+---------
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L  P  W  T  Q  V  V  G  A  F  P  G
```

PRV gIII

```
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      M  A  S  L  A  R  A  M  L  A  L  L  A  L  Y  A  A  A

                        BamHI
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 I  A  A  A  P  S  G
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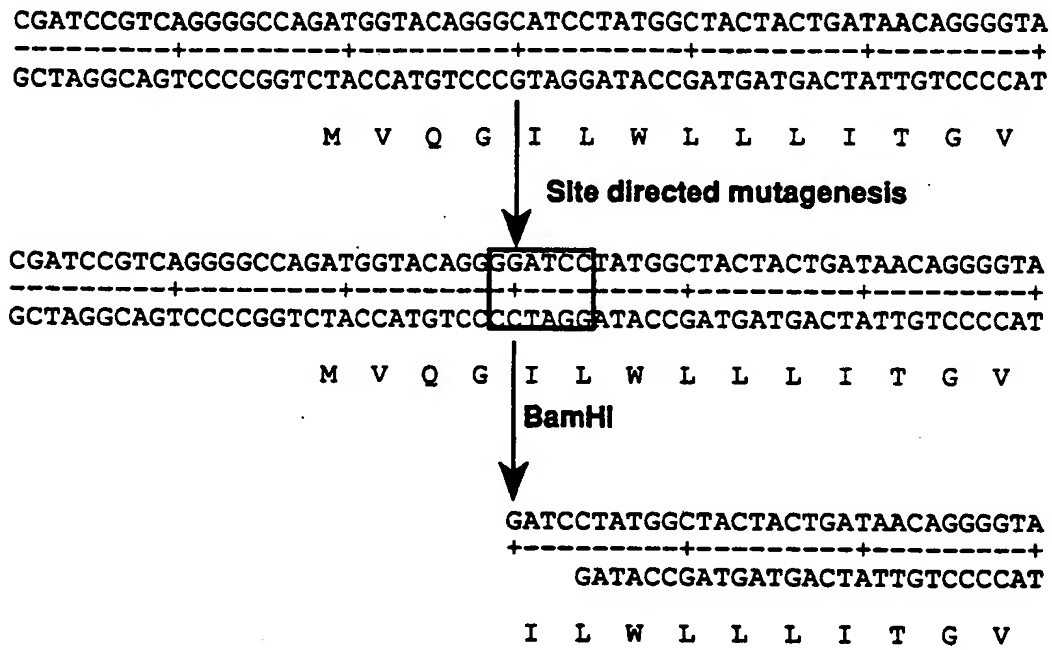
FIGURE 2B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Site directed mutagenesis
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BamHI
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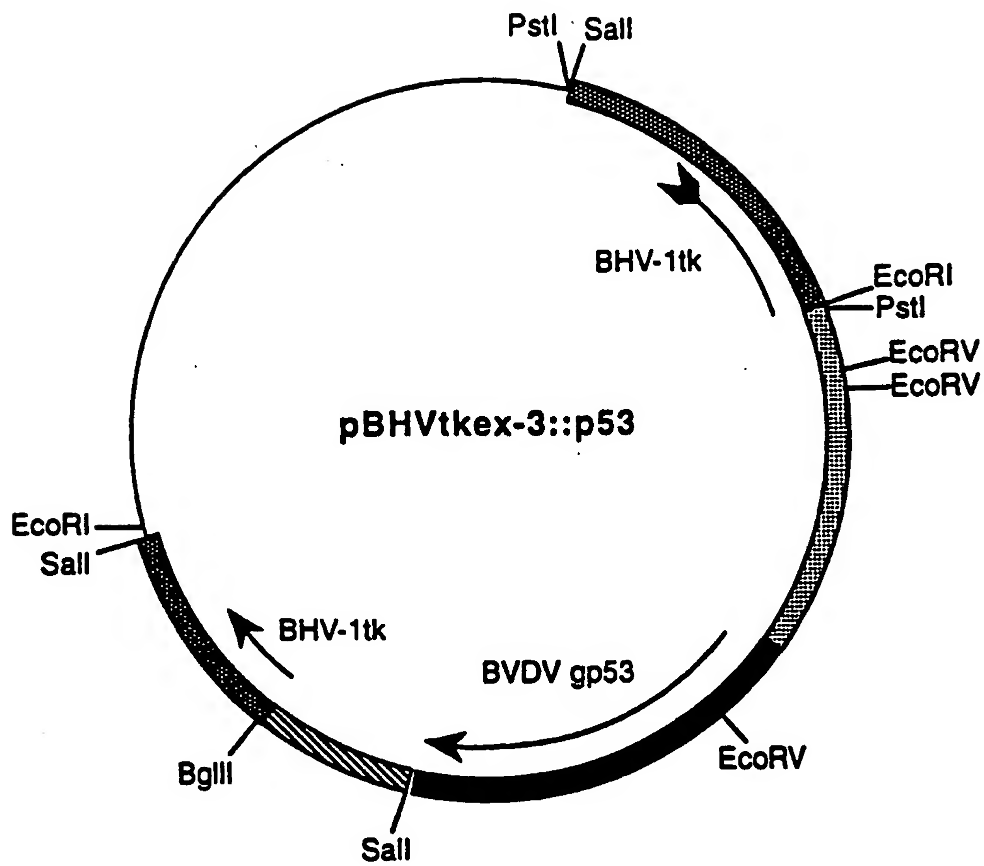
I L W L L L I T G V

FIGURE 3A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SalI
BHV-1tk
EcoRI
PstI
EcoRV
EcoRV
pBHVtkex-3::p53
EcoRI
SalI
BHV-1tk
BVDV gp53
EcoRV
BglII
SalI

FIGURE 3B

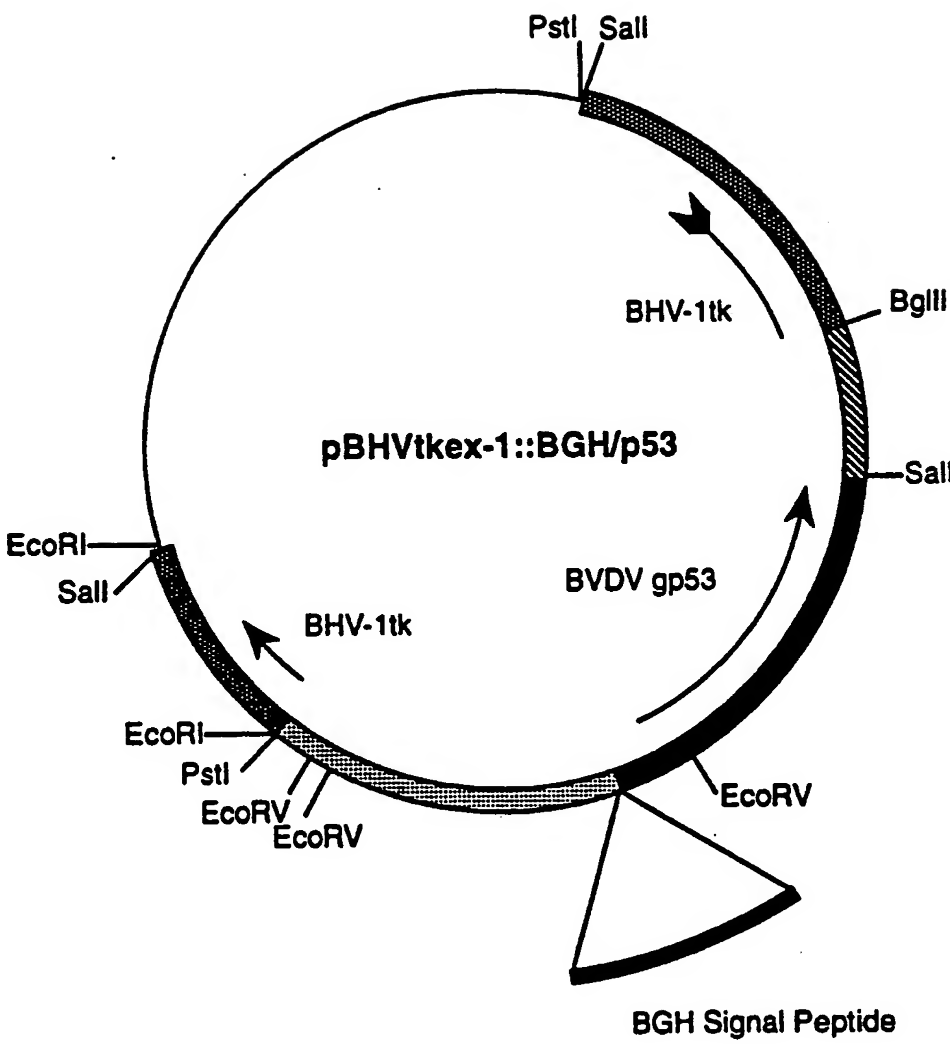

PstI
SalI
BglII
SalI
BHV-1tk
pBHVtkex-1::BGH/p53
BVDV gp53
EcoRI
SalI
BHV-1tk
EcoRI
PstI
EcoRV
EcoRV
EcoRV
BGH Signal Peptide

FIGURE 3C

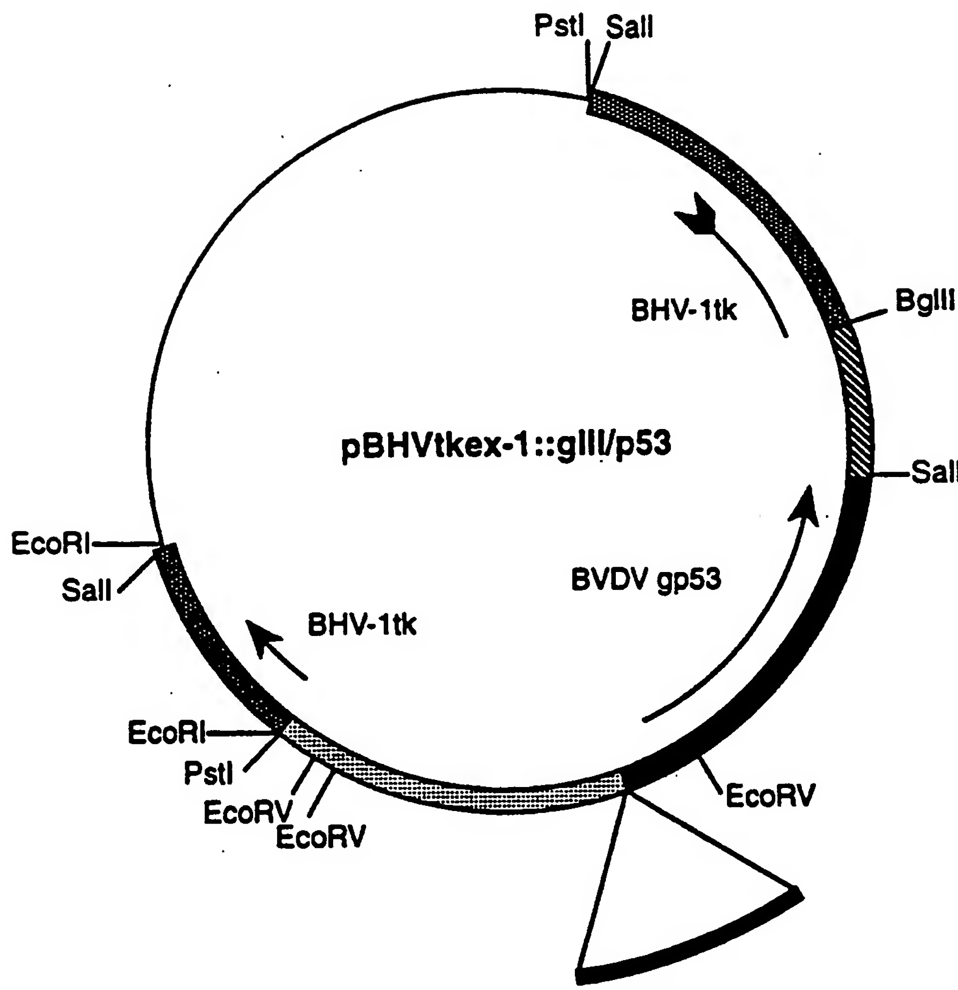
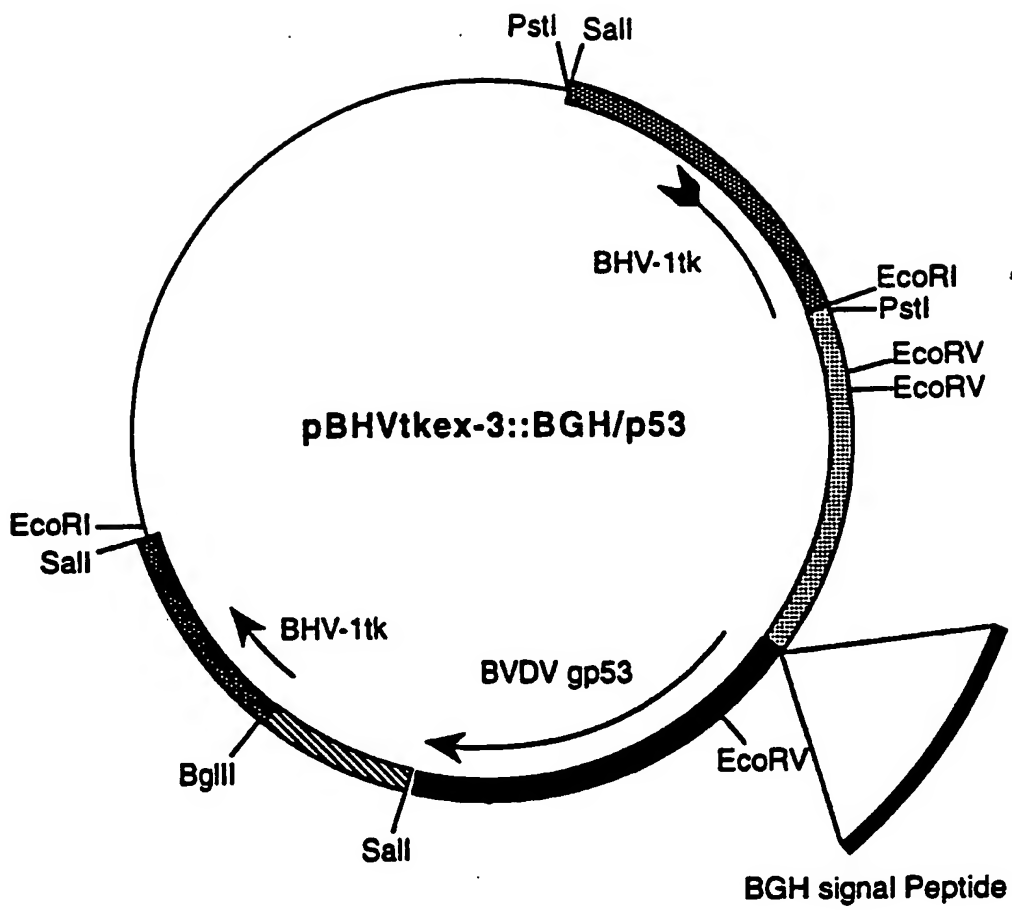

PstI
SalI
BHV-1tk
BglII
pBHVtkex-1::gIII/p53
SalI
EcoRI
SalI
BVDV gp53
BHV-1tk
EcoRI
PstI
EcoRV
EcoRV
EcoRV
PRV gIII Signal Peptide

FIGURE 3D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SalI
BHV-1tk
EcoRI
PstI
EcoRV
EcoRV
pBHVtkex-3::BGH/p53
EcoRI
SalI
BHV-1tk
BVDV gp53
EcoRV
BglII
SalI
BGH signal Peptide

FIGURE 3E

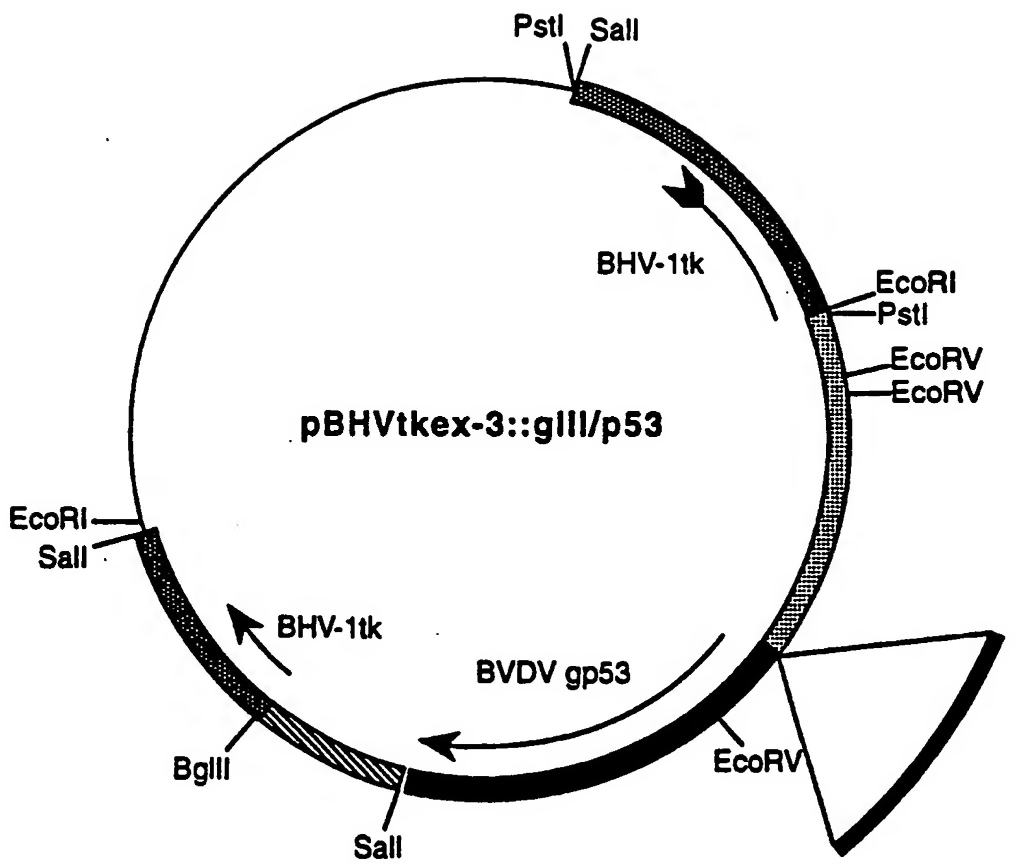
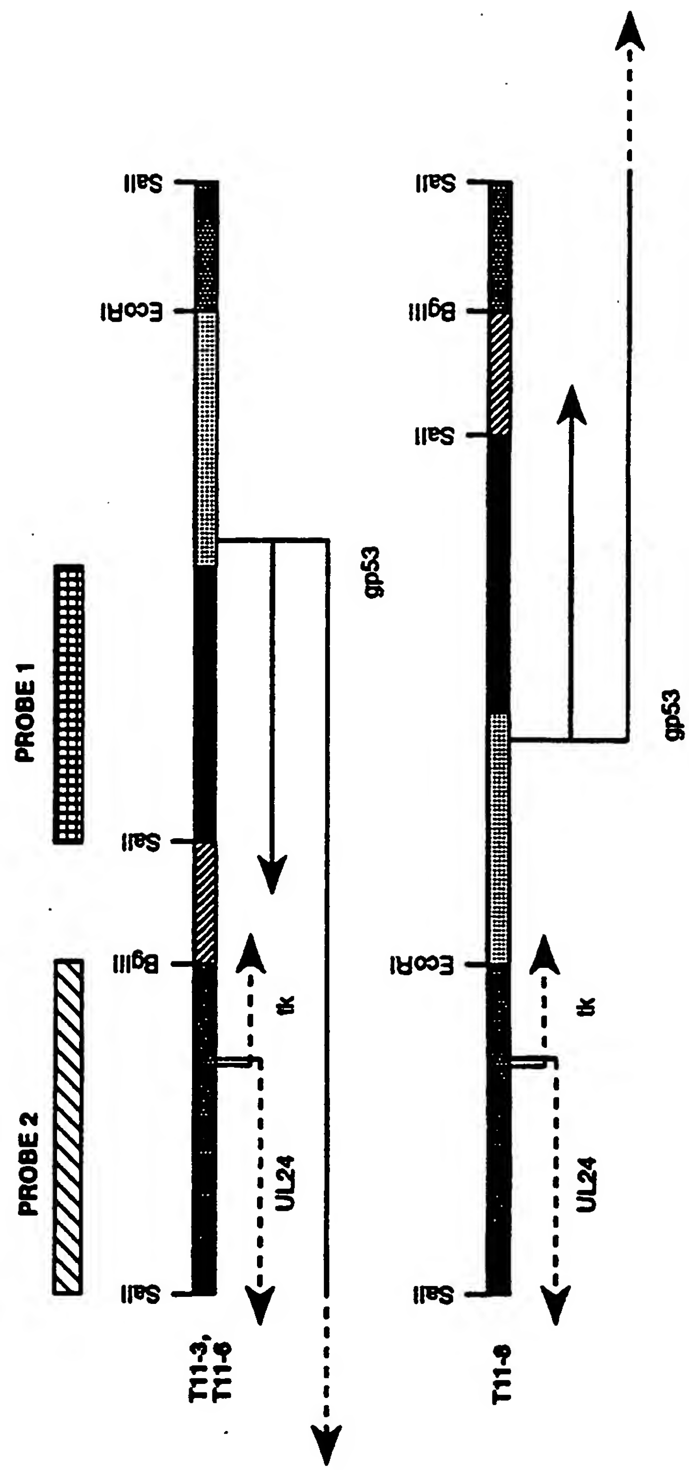

PstI
SalI
BHV-1tk
EcoRI
PstI
EcoRV
EcoRV
pBHVtkex-3::gIII/p53
EcoRI
SalI
BHV-1tk
BVDV gp53
EcoRV
BglII
SalI
PRV gIII signal Peptide

FIGURE 4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EcoRI
SalI
BglII
SalI
PROBE 1
PROBE 2
gp53
tk
UL24
T11-3,
T11-6
SalI
BglII
SalI
EcoRI
SalI
gp53
tk
UL24
T11-8

FIGURE 5A

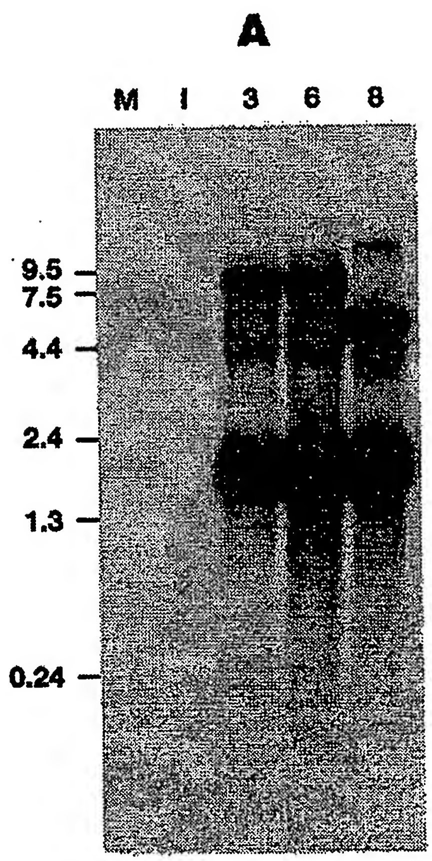


FIGURE 5B

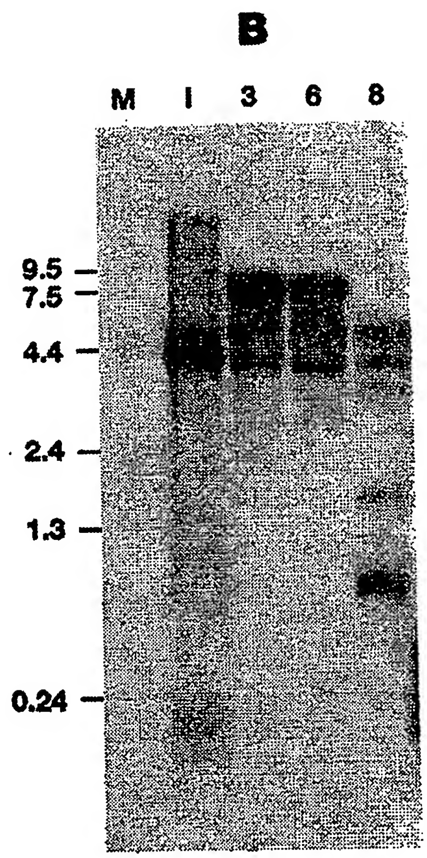
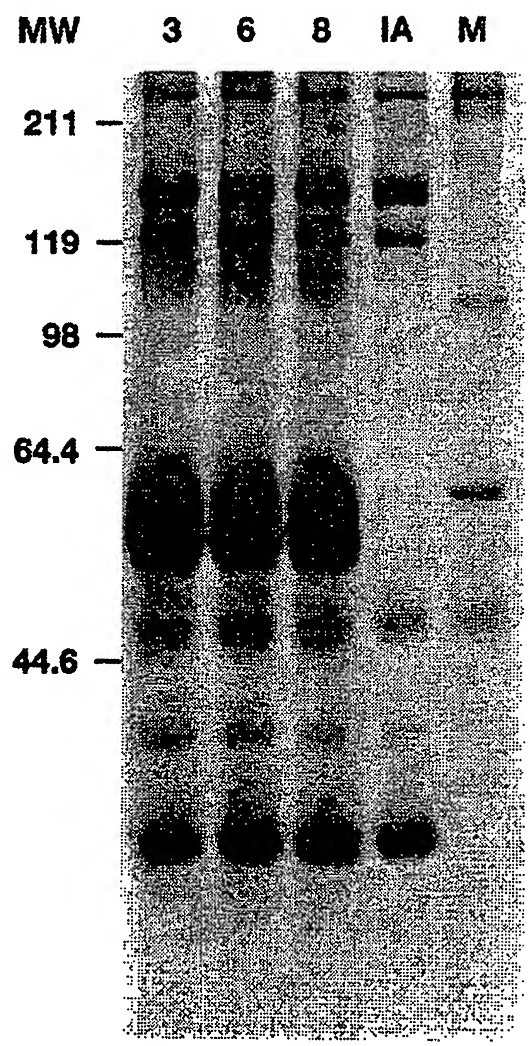


FIGURE 6



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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).

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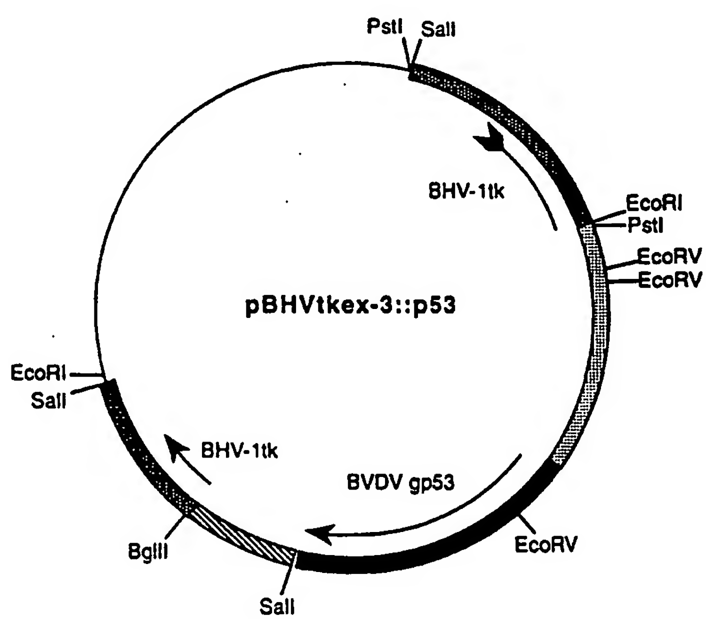

(57) Abstract

This invention relates to the field of Bovine Viral Diarrhea Virus (BVDV), and vaccines for the treatment thereof. This invention describes the preparation of live, attenuated Bovine Herpesvirus type 1 (BHV-1) as a virus, vaccine and vector for expression of BVDV antigens. A BVDV cDNA clone containing sequences corresponding to glycoprotein gp53 is inserted into an inactivated BHV-1 virus.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/L_ 94/12198

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C07K14/18 C12N7/01 A61K39/12 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,94 00586 (RHONE-MERIEUX) 6 January 1994 see claim 11 ---	1-3,25, 27,28
Y	VIROLOGY, vol.190, no.2, 1992 pages 666 - 673 BELLO, L. ET AL. 'Bovine herpesvirus 1 as a live virus vector for expression of foreign genes' see the whole document --- -/--	1-3,6,7, 25,27-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
19 May 1995	07.06.95

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VIROLOGY, vol.190, no.2, 1992 pages 763 - 772 PATON, D.J. ET AL. 'Epitope mapping of the gp53 envelope protein of bovine viral diarrhea virus' see the whole document ---	1-3,6,7, 25,27-30
P,A	VIRUS RESEARCH, vol.34, no.2, 1994 pages 178 - 186 YU, M. ET AL. 'High level expression of the envelope glycoprotein (GP53) of bovine viral diarrhoea virus (singer) and its potential use as diagnostic reagent' see the whole document ---	1
A	EP,A,0 464 010 (STATENS VETERINÄRMEDICINSKA ANSTALT) 2 January 1992 see the whole document ---	1
A	WO,A,90 01337 (INSTITUTE FOR ANIMAL HEALTH LIMITED) 22 February 1990 see the whole document ---	1
A	EP,A,0 119 025 (BAYLOR COLLEGE OF MEDECINE. NOVAGENE LTD) 19 September 1984 see the whole document -----	1

1

INTERNATIONAL SEARCH REPORT

..ternational application No.
PCT/US94/12198

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 27 is directed to a method of treatment of the animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

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International Application No
PCT/.. 94/12198

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9400586	06-01-94	FR-A- 2693472	14-01-94
		AU-B- 4334193	24-01-94
		CA-A- 2116355	06-01-94
		EP-A- 0605680	13-07-94
EP-A-0464010	02-01-92	NONE	
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		CA-A- 1319634	29-06-93
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		GB-A- 2239799	17-07-91
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		US-A- 4569840	11-02-86